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(54) Material and process for immunological assay

(57) Antigen- or antibody-bound microcapsules having a core and a wall with an antigen or antibody bonded thereto are employed as a material for immunoassay of biochemical components. The microcapsules provide high detection sensitivity, high accuracy and excellent reproducibility in immunological assay, without causing any non-specific reaction.

These microcapsules can be labelled with isotopes, enzymes, fluorescent or magnetic substances, ultraviolet absorbents or dyes, for use in quantitative assay of a trace amount of the biochemical components.

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SPECIFICATION

Novel material for immunological assay of biochemical components and a process for the determination of said components

The present invention relates to microcapsules for immunological determination of a trace amount of biochemical components utilizing an immunological reaction, which microcapsules comprise a core material and a wall material having an antigen or an antibody bond on the surface thereof.

The present invention also relates to a process for the determination of such biochemical components using the microcapsules of the invention.

Various immunological methods have been heretofore proposed for the determination of biochemical components having immunological activity. For example, one proposed method for determining biochemical components comprises observing, on a microplate or the like, agglutination occuring between test samples and antigen- or antibody-coupled particles, such as animal red cells also referred to as enythrocytes, high molecular weight latex, high molecular weight polymer particles, etc. In recent years, attention has been focused on radioimmunoassay and enzyme immunoassay.

In the prior art methods, sheep red cells are most commonly used as carrier particles for binding the antigen or antibody used in agglutination of an antigen-antibody reaction. Organic or inorganic particles such as polystyrene latex, polyester, nylon, kaolin, etc. have also been employed. When used as carrier particles, red cells have previously been coupled with an antigen or antibody chemically using an aldehyde such as glutaraldehyde, and polystyrene latex, polyester, nylon, kaolin, etc. have been physically bound thereto utilizing physical adsorption.

However, when using sheep red cells as carrier particles, quality is uneven and antigen-antibody reaction can be only interpreted with poor accuracy since these carrier particles originate from the living body. Furthermore, non-specific reactions tend to occur since the sheep red cell carrier *per se* has antigens and antibodies in nature, and therefore reproducibility of antigen-antibody reaction is low and the cost is high. There are disadvantages when using sheep red cells as carrier particles. On the other hand, the systems using polystyrene latex, polyester, nylon, inorganic particles or the like involve the shortcomings that, in addition to expense, immobilization is because of the adsorption bonding of an antigen or antibody on the carrier particles and, as a result, the antigen or antibody is easy to isolate and the sensitivity of the antigen-antibody reaction is reduced and reproducibility of the antigen-antibody reaction is low. These disadvantages are particularly noticeable especially when the materials have been stored over a long period of time.

This invention provides microcapsules for use in immunological determination of biochemical components which overcome the prior art disadvantages.

The invention also provides a process for determination of biochemical components immunologically using such microcapsules.

In the present invention, the term "bind, bonding or bound, etc." refers to a state of attaching an antigen or antibody directly or indirectly to the surface of a wall material of microcapsules in a broad sense. So long as the antigen- or antibody- "bound" microcapsules effectively cause an immunological response or reaction, the method of bonding is immaterial. However, in a general usage in this art, an antigen or antibody is bound to a microcapsule wall by means of cross-linking, covalent binding or coupling and the term "bind, binding or bound" used in the specification collectively refers to all of these cases.

The term "antigen" is established in the immunological art and, phrased another way, means lipids, proteins, glucoses and complexes thereof (e.g., glucoproteins, lipoproteins, glucolipids) having immunogenicity. The term "antibody" is also established in the art and no further explanation is needed; however, antibodies are also collectively called immunoglobulins.

According to the invention, a trace amount of components contained in a sample to be examined can be detected easily and accurately in a simple manner.

The microcapsules which can be employed in the invention comprise a core material and a wall material having an antigen or antibody bonded to the surface of the wall material.

For binding an antigen or antibody to the microcapsules, various conventional methods may be employed, e.g., an aldehyde cross linking method, a cyanbromide method, a carbodiimide cross linking method, an alkylation method, an isocyanate cross linking method, a maleimide cross linking method, a benzophenone cross linking method, a periodic acid cross linking method etc., the details of which are described in Ichiro Chihata, *KOTEIKA KOSO* (Immobilized Enzyme), Kodansha Publishing Co., Ltd., (1975), and Eiji Ishikawa, *KOSO MENEKI SOKUTEIHO* (Enzyme Immunoassay), p. 34 to 44. While the binding methods referred to are not limitative, it is important that the antigens or antibodies are bound in such a way that they are not inactivated. The most typical method is binding using aldehydes such as glutaraldehyde formaldehyde, glyoxal, etc. According to this method, the microcapsules are mixed with 0.2 to

2% of, e.g. glutaraldehyde at temperatures of 15 to 40°C, preferably ambient temperature, for 1 to 2 hours, under normal pressure and the mixture is then washed with distilled water to remove the unreacted aldehyde. Next, the glutaraldehyde-treated microcapsules are mixed with 0.1 to 5% antigen or antibody, and reacted at room temperature for 1 to 2 hours. Specific examples of cross linking reagents other than aldehydes include toluene–2,4–diisocyanate, N,N',O–phenylenedimaleimide, m-maleimidebenzoyl, N-hydroxysuccinimide ester, etc.

In order to check if a bond is formed between the microcapsules and the antigen or antibody, a simple test can be generally performed by an immunofluorescene technique (*A Dictionary of Immunology*, pages 128 and 129 (1979), published by Hirokawa Publishing Co., Tokyo) which comprises bonding a fluorochrome to the antibody, mixing the fluorochrome-labelled antibody with microcapsules to be bound to the antigen and then measuring the fluorochrome after washing the mixture with water. If the fluorochrome can still be detected in the mixture, it is judged that the microcapsule-antibody bond will be effective, and *vice versa*.

When bonding the antigens or antibodies to the microcapsules, it is neccesary to choose the composition of microcapsule walls to suit the combination of antigens or antibodies and the wall material. For example, it is advantageous in binding antigens or antibodies to microcapsule walls using aldehydes that active protons such as amino, imino, hydroxy groups etc be present in the microcapusle walls. One skilled in the art can easily determine which combination is suitable for binding antigens or antibodies to microcapsule walls and no further explanation is needed.

Any material can be used as the wall of the microcapsules in the present invention provided the material can bind to antigens or antibodies without inactivating the antigens and antibodies and enable encapsulating them. Represnetative examples of wall materials are materials having an amino, imino, hydroxy or sulfhydryl group such as proteins (e.g., collagen, gelatin, casein etc.); resins such as polyamino acid, polyacrylamide, polyamide, polyurethane, polyurea, polyurethane-urea, melamine resin, phenol resin, epoxy resin, silicone resin and derivatives thereof; cellulose and derivatives thereof (e.g. methyl cellulose, ethyl cellulose, carboxymethyl cellulose, nitrocellulose, cellulose acetate, cellulose sulfate, etc.), gum arabic, starch, alginic acid and the like.

Advantageously, the average size of the microcapsules ranges from about 0.1 to about 30 μ, preferably 0.5 to 10 μ.

Details of various wall materials and method for microencapsulation are described in e.g., Asaji Kondo, *MICROCAPSULES*, Nikkan Kogyo Press, Tokyo (1970), Tamotsu Kondo and Masumi Koishi, *MICROCAPSULES*, Sankyo Publishing Co., Ltd., Tokyo (1972), etc.

Typical examples of oleophilic substances which can be core materials for the capsules are natural mineral oils, animal oils, vegetable oils and synthetic oils.

Specific examples of mineral oils are petroleum, kerosene, gasoline, naphtha, paraffin oil, etc. Animal oils typically include fish oil, lard, etc. Typical examples of vegetable oils include peanut oil, linseed oil, soybean oil, castor oil, corn oil, etc. Specific examples of synthetic oils are biphenyl compounds (e.g., isopropyl biphenyl, isoamyl biphenyl), terphenyl compounds (e.g., see OLS 2,153,635), naphthalene compounds (e.g., diisopropylnaphtalene, U.S. Patent 4,003,589), alkylated diphenylalkanes (e.g., 2,4-dimethyldiphenylmethane, U.S. Patent 3,836,383), phthalic acid compounds (e.g., diethyl phthalate, dibutyl phthalate, dioctyl phthalate), etc.

The core material present in the microcapsules of the invention is not limited to the substances described above.

In order to improve contrast when using the microcapsules in agglutination processes, oleophilic coloring dyes may be incorporated in the core material. As coloring dyes there may be mentioned for example, Color Index Solvent Red 1, 3, 8, 23, 24, 25, 27, 30, 49, 81, 82, 83, 84, 100, 109, 121, Color Index Solvent Violet 8, 13, 14, 21 and 27; Color Index Solvent Blue 2, 11, 12, 25, 35, 36, 55 and 73; Color Index Solvent Green 3; Color Index Solvent Brown 3, 5, 20 and 37; Color Index Solvent Black 3, 5, 7, 22, 23 and 123, etc. all of which can be employed. Although it is possible to color carrier particles in order to improve contrast particularly in antigen-antibody agglutination, when using e.g., polystyrene latex, polyester, nylon, etc., as in the prior art the dyes which are available are extremely limited since there are many chances that dyes might adversely affect the antigen-antibody reaction. The choice of dyes can thus be troublesome which causes high production cost. On the other hand, when using microcapsules as carrier particles as in the invention, it is sufficient to incorporate the dye into the core material so that the chance of an adverse effect on the antigen-antibody reaction is reduced. Thus, according to the invention it is possible to choose any dyes which improves contrast and reduces cost. When using sheep red cells specific gravity and particle size cannot be exactly controlled since it is natural substance, and this control is also difficult when using polystyrene latex, polyester, nylon, inorganic particles, etc. However, when using microcapsules as carrier particles, specific gravity can easily be controlled by mixing two kinds of core materials having different specific gravities, or by dispersing inactive solid particles in the core material, etc. When using microcapsules particle size can also easily be controlled. For these reasons, the

use of microcapsules as carrier particles is advantageous.

From the viewpoints of immediate antigen-antibody response and improvement in sensitivity, it is desirable that the specific gravity of the microcapsules ranges from about 0.8 to about 1.20. When the specific gravity range is set between about 1.05 and about 1.20, preferably between about 1.07 and about 1.16, the system can advantageously be used in measurement of an antigen or antibody by means of a micro titer method (passive agglutination method using a microplate). If microcapsules having a specific gravity between about 1.11 and about 1.15 are utilized, not only is the time period necessary for judgement of a sample to be examined, i.e., the time period until a difference appears on the microplate between a positive and negative pattern, which period is herein referred to as "judgement time" markedly shortened, but also a positive pattern or negative pattern appears extremely clearly. In addition, when the specific gravity of the microcapsules approximates 0.8, agglutination can easily be performed using whole blood without separating red cells from the blood. Further, as compared to the prior art system using sheep red cells as carrier particles, the microcapsule system of the invention does not show undesired non-specific reactions which are unavoidably observed when using naturally occurring sheep red cells. The microcapsule system is also excellent since it has the property of stable storage over a long period of time.

The microcapsule system according to the invention can be utilized in a wide variety of immunological examinations and this utility is further extended to a broad area if the microcapsule system is labelled with marking substances. The microcapsule system of the invention is advantageously applicable to radioimmunoassay which comprises determining an unknown substance by causing antigen-antibody reaction between the unknown substance and the microcapsule system labelled with radio isotope such as I^{125}, I^{131}, H^3, C^{14}, etc (details are described in Kumahara and Shizume, *Shinpan Radioimmunoassay*, pages 3 to 10, 1977, published by Asakura Shoten, Tokyo, Etc.).

The amount of isotopes to be labelled varies depending upon kind of the microcapsule system used, antigen or antibody, etc. and is not generally limitative; however, isotopes having an intensity of 100 mci/ml are generally used and are effective in achieving marking purposes. Other marking substances useful to determine unknown substances are described below.

The marking substances which can be advantageously employed in the present invention are e.g., isotopes, enzymes, fluorescent substances, magnetic substances, ultraviolet absorbents, dyes, etc. These marking substances can suitably be employed depending upon content of an unknown substance to be determined so that detection sensitivity can be enhanced. Of these marking substances, substances other than isotopes are preferred in view of problems of waste matter treatment, operational safety and storage of the marking substances. In addition, such substances provide higher sensitivity for detection and lower cost in a simpler manner than in the case when using isotopes.

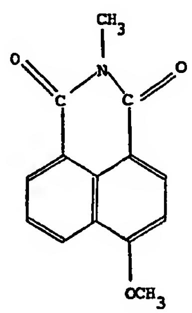
These marking substances can be bound to the outside (surface) of the wall material of the microcapsules or incorporated into the core material thereof. When the marking substances are incorporated (or dispersed) in the core material, there is a large tolerance for choosing the marking substances and the content thereof so that it is possible to select appropriate marking substances depending upon their purpose. In addition, it is also possible to effectively proceed with immunological reaction since the marking substances are not brought into direct contact with an antigen or antibody. For instance, enzyme immunoassay has heretofore encountered disadvantages that storability and stability are poor as a marking substance and the method per se is unsatisfactory in accuracy and reproducibility. However, in the case of using enzyme incorporated into the core material which consists of an aqueous solution of the microcapsule system of the present invention, such disadvantages encountered in the prior art can be overcome.

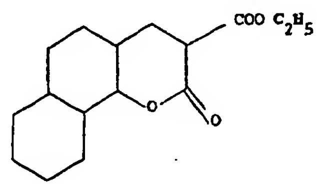
As a representative of directly labelling antigens or antibodies with fluorescent substances, a method of using FITC Anami, etal, *KISO SEIKAGAKU JIKKENHO* (Basic Biochemical Experiment), Vol. 6 P. 167 (1976), Maruzen, Tokyo is known. However, according to this method the amount of fluorescent substances that can be attached to molecules of antigens or antibodies is limited so as not to inhibit immune reaction. Therefore, in order to further enhance the sensitivity, it is desired that fluorescent substances be incorporated into the core material. In this embodiment, marking substances can be used in an amount required for the detection sensitivity required so that an extremely high marking intensity can be obtained and hence the detection sensitivity can be markedly enhanced. Measurement of an extreme trace amounts of antigens or antibodies contained in a sample to be examined can be made by binding antigens or antibodies corresponding to components in the sample onto the surface of such microcapsules having incorporated therein, e.g., fluorescent substances, causing immune reaction between the microcapsules and antigens or antibodies contained in the sample directly or indirectly and then separating the antigen-antibody reaction product from the unreacted antigens or antibodies, and determining an amount of fluorescent substance contained in the microcapsules. In the system where marking substances are microencapsulated in a core material as

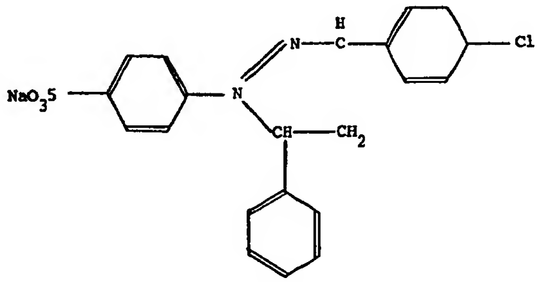
described above, a material for immunological assay having improved stability, accuracy and reproducibility and high sensitivity can be provided due to excellent uniformity of the core material and improved coating ability of the wall material.

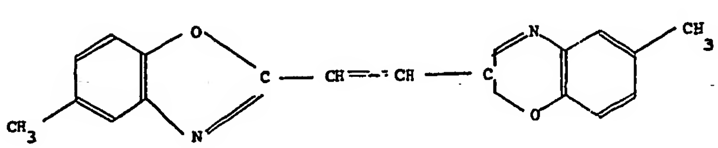
Further, the microcapsules of the present invention can be used as carriers upon immunological response. For example, the use of carriers for radioimmunoassay (*A Dictionary of Immunology*, P.290 (1979), Hirokawa Shoten, Tokyo) and enzyme immunoassay (see E.Ishikawa, *Enzymeimmunoassay*, 1978, Igakushoin, Tokyo) is representative. Furthermore, the microcapsules of the present invention can be employed instead of blood cells such as ènythrocyte, lencocyte, lymphocyte, or glass beads conventionally used for determining biological components contained in the sample.

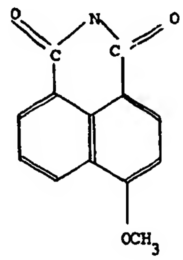
Typical examples of fluorescent marking substances which can be used in the present invention include stilbene and derivatives thereof (e.g., 4,4'–diaminostilbene derivatives, 4,4'–diaminostilbene disulfonic acid derivatives, aminostilbene–2,2'–disulfonic acid derivatives, etc.), coumarine derivatives, benzoxazole derivatives, bisoxazole, pyrazoline derivatives, 4,4'–bistriazinyl, etc., which are exemplified by the following compounds.

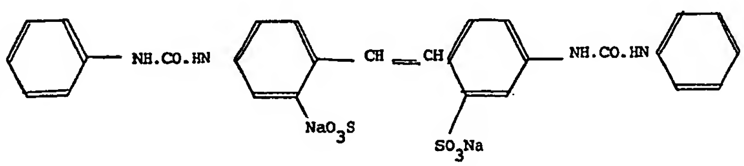












Specific examples of enzymes which can be employed as marking substances in the present invention include alkali phosphatase, β-galactosidase, acetylcholine esterase, glucoamilase, maleic acid dehydrogenase, glucose-6-phosphoric acid dehydrogenase, peroxidase, glutaroxidase, etc.

Specific examples of magnetic substances which can be employed as marking substances in the present invention include iron powders, nickel, cobalt, CrO_2, CoO, NiO, Mn_2O_3, magnetic zinc oxides, magnetic iron oxides such as $MnFe_2O_4$ powders, Fe_3O_4 powders, $CoFe_2O_4$ powders, $NiFe_2O_4$ powders, $CuFe_2O_4$ powders, $MgFe_2O_4$ powders, etc.; alloys of Al, Ni, Co, Cu, etc.; non-crystalline substances having magnetic property such as calcogenides, ferricolloid powders, etc.

Specific examples of UV absorbents which can be employed as marking substances in the present invention include salicylic acid derivatives, e.g., phenyl salicylate, 4-t-butylphenyl salicylate, bisphenyl A-disalicylate, etc.; benzophenone derivatives, e.g., 2-hydroxy-4-methoxybenzophenone, 2-hydroxy-4-methoxy-2'-carboxybenzophenone, 2-hydroxy-4-octoxybenzophenone, 2-hydroxy-4-octoxybenzophenone, 2-hydroxy-4-stearlyoxybenzophenone, 4-dodecyloxy-2-hydroxybenzophenone, 2-hydroxy-4-methoxy-5-sulfobenzophenone trihydrate, 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid, 2-hydroxy-4-(2-hydroxy-3-methacryloxy)-propoxybenzophenone, 2-hydroxy-4-methoxy-4'-methylbenzophenone, 2-hydroxy-4-benzoyloxybenzophenone, 2,4-dihydroxybenzophenone, 2,2'-dihydroxy-4-methoxybenzophenone, 2,2'-dihydroxy-4-n-octoxybenzophenone, methyl o-benzoylbenzoate, 2,2',4,4'-tetrahydroxybenzophenone, etc.; benzotriazole derivatives such as 2-(2'-hydroxy-5'-methylphenyl)-benzotriazole, 2-(2'-hydroxy-3'-t-butyl-5'-methylphenyl)-5-chlorobenzotriazole, 2-(2'-hydroxy-3',5'-di-t-butylphenyl)-5-chlorobenzotriazole, 2-(2'-hydroxy-3',5'-dibenzylphenyl)-benzotriazole, 2-(2'-hydroxy-4'-octoxyphenylbenzotriazole, etc.

In the present invention, dyes can also be employed as markers. Typical examples of such dyes are those previously exemplified for use in incorporating into the core material for the purpose of coloring the microcapsules.

In quantitatively determining the labelled substance, suitable methods or means for the marking substances employed can be chosen. For example. when using fluorescent marking substances, an amount of fluorescene is determined with a fluorophotometer, fluoropolarization photometer, etc. When using magnetic marking substances, electromagnetic properties can be measured to determine the labelled substances. Further when using UV absorbents, quantitative determination can be made with a spectrometer. In measurement of turbidity or transmittance, or qualitative determination of the marking substances, lasers can be advantageously employed.

In general prior to the quantitative determination, the microcapsules are mechanically mashed as a pretreatment.

The amount of the markers to be used are generally between about 0.1 and about 10 wt% based on the core material used, preferably 0.5 to 5 wt% when fluorescent substances, magnetic substances, UV absorbents and dyes are used as the markers. When enzymes are used, the amount is generally between about 10^{-2} and 10^{-10} mole per 1 g of the core material, preferably 10^{-6} to 10^{-10} mol.

In the preferred embodiment of the present invention, the marking substances are microencapsulated as a core material so that the marking substances are not adversely affected by various substances present outside the microcapsule walls. In addition, adverse influence by various conditions outside the microcapsule walls, e.g., pH or temperature, can be prevented. Accuracy can also be improved in qualitative assay due to uniform and accurate encapsulation of the marking substances in microcapsules. Encapsulation of the marking substances in microcapsules in an amount required results in marked enhancement of sensitivity of detection.

The antigen- or antibody-bound microcapsules of the present invention can be utilized in various immunological reactions. Firstly, the microcapsule system can be effectively utilized in agglutination, more specifically, a glass plate method (antigen- or antibody-bound microcapsules are mixed with a sample to be examined on a glass plate. Biological components can be detected whether or not agglutination occurs.), a microplate method (which is also referred to as a microtiter method; double-double serial diluents of a sample to be examined are charged into holes of a V- or U-shape having a diameter of about 5 m/m and the antigen- or antibody-bound microcapsules are dropped thereon. Qualitative assay can be made by determining at which concentration agglutination occurs.).

In the present invention, antigen-antibody reaction is carried out in conventional manner, e.g., a direct antibody method, a direct antigen method, an indirect antibody method, a sandwich method, an indirect complement method, a direct complement method, etc., details of which are described in M. Kaneizumi, *RINSHO KENSA GAIYO* (outline of Clinical Test), Vol XX, Page 10, Kanehara Publishing Co., 1978.

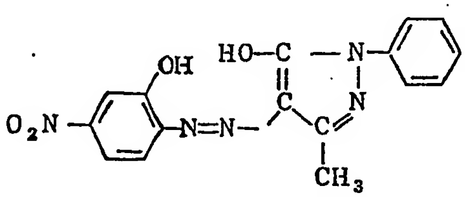
Biological components which can be determined according to the present invention are e.g., peptide hormones such as hypothalamus hormones (e.g., TRH, LH–RH, somatostatin), hypophysis hormones (e.g., growth hormone, ACTH, α-MSH, β-MSH, lipotropin, prolactin, TSH, TSH–β, LH, LH-β, FSH, FSH-β, α-subunit, arginine vasopressin, lysine vasopressin, oxytocin, etc.), calcium metabolism regulating hormones (e.g., calcitonin, parathormone, etc), pancreas hormones (e.g., insulin, proinsulin, C-peptide, glucagon, etc.), digestive tract hormones (e.g., gastrin, secretin, pancreozymin-cholecystokinin, GIP, enteroglucagon, etc.), hormones acting on blood vessels (e.g., antiotension I, angiotension II, bradykinins, etc.), placenta hormones (e.g., human chorionic gonadotropin (HCG), HCG-β, human chorionic somatomammotropin, human chorionic thyrotropin; non-peptide hormones such as steriods (e.g., cortisol, corticosterone, 11–deoxycortisol, 11–deoxycorticosterone, progesterone, 17–hydroxyprogesterone, pregnenolone, aldosterone, testosterone, dihydrotestosterone, estradiol, estriol, estrone, 2–hydroxyestrone, dehydroepiandrosterone, medorxyprogesterone, etc.), thryoid hormones (e.g., thyroxine, 3,5,3′-triiodothyronine, 3,3′5′-triiodothyronine, etc), prostaglandins (e.g., prostaglandin A, E, F, etc.); substances other than hormones such as drugs (e.g., digoxin, digitoxin, morphine, LSD, gentamycin, amphetamine, nicotine, cotinine, etc.), cyclic nucleotides (e.g., cyclic AMP, cyclic GMP, cyclic IMP, cyclic UMP, etc.), enzymes (e.g., C_1 esterase, fructose 1,6–diphosphatase, alkaline phosphatase, dopamine beta hydroxylase, pepsinogen, etc.), virus specific antigens (e.g., hepatitis B Virus, murine sarcomaleukemia virus, wooly monkey leukemia virus, avian tumor virus, plant virus, avian C-type virus, etc.), tumor antigens (e.g., α-fetoprotein, carinoembryonic antigen (CEA), etc.), blood serum proteins (e.g., thyroxine binding glubulin (TBG), IgG, IgM, IgE, IgA, α_2–microglobulin, properdin, anti-Rh antibodies, transferrin, aplipoprotein, fibrinogen degradation products, antihemolytic factor, renin, etc.); rheumatism factor, folic acid, neurophysin, somatomedin B, nerve growth factor, epidermal growth factor, staphylococcal enterotoxin A and B, type A toxin of clostridium botulinum, myosin, encephalitogenic basic proteins, substance P, serotonin, conjugated cholyl bile acid, H_{85}–antigen, etc. Needless to say, the biological components which can be determined in accordance with the present invention are not limited thereto.

According to the present invention, it is sufficient for determination of biological components that the content be 10^{-9} g/ml in a sample to be examined.

In the following examples the sensitivity for detecting an antibody is expressed as the minimum antibody concentration at which the antigen-bound microcapsules cause agglutination. This minimum antibody concentration is obtained by mixing double-double serial diluents of the antibody with the antigen-bound microcapsule solution at a fixed concentration and observing the minimum concentration of the antibody at which agglutination is caused.

Example 1

In 40 parts of warm water at 40°C., 5 parts of acid-treated gelatin having an isoelectric point of 7.8 and 5 parts of gum arabic were dissolved. While vigorously stirring, 50 parts of a diisopropylnaphthalene/chlorinated paraffin (cholination degree, 50%) oil mixture (mixing ratio = 23.6:26.4) having a specific gravity of 1.10 and containing 1% of a dye of formula:



were added to the solution to obtain an O/W emulsion having an average drop size of 6.0 μ. Thereafter, the emulsion was diluted by adding 213 parts of warm water of 40°C. thereto.

Then, acetic acid was dropwise added to the emulsion under a fixed rate stirring to reduce pH of the system to 4.6 and thereby cause coacervation.

After the system was cooled to 10°C. to gel the coacervate, the system was hardened by the addition of 2 parts of 37% formaldehyde. Then, 40 parts of a 10% aqueous solution of carboxymethyl cellulose (average polymerization degree; 220) were added to the system. In order to enhance the hardening effect, a 10% aqueous sodium hydroxide solution was dropwise added to adjust the pH to 10. The temperature of the system was further raised to 50°C. The thus prepared microcapsules were washed with water and filtered to remove the remaining formaldehyde. The microcapsules had a specific gravity of 1.10 and an average particle size of 6.3 μ.

The thus obtained microcapsules were then washed with a phosphoric acid buffer (an aqueous solution obtained by dissolving 8 g. of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4.12H_2O$ and 0.2 g of KH_2PO_4 in water to make 1 1.). 0.5 g. of microcapsules was dispersed in 5ml. of the phosphoric acid buffer. To the resulting dispersion, 1ml. of egg albumin (10 mg/ml) was added as antigen. Subsequently, 100 μl of a 25% aqueous glutaraldehyde was added to the mixture. The mixture was reacted for 1 hr. at room temperature. Thereafter, the reaction mixture was centrifuged and washed with the phosphoric acid buffer 0.5 g. of reaction mixture was taken and again dispersed in 5 ml. of the phosphoric acid buffer. Thus, the buffer containing 1% of the antigen-bound microcapsules were obtained (sample #1).

Next, the phosphoric acid buffer was dropped with a dropper one drop (25 μl) in each well of the two lines on a microplate having 12 wells x 2 lines.

Then, 25 μl of rabbit antisera to egg albumin was taken with a diluter and added to the dilution liquid (phosphoric acid buffer) on the first line (1/2 dilution). After sufficiently stirring, 25 μl was taken from the first diluent well and added to a second well on the first line (1/4 dilution) followed by sufficient stirring. Again, 25 μl was taken from the second diluent well and added to a third well on the first line followed by sufficient stirring (1/8 dilution). These procedures were repeated to obtain 2^{12} (4096) time dilutions.

Further, 25 μl of 1% antigen-binding microcapsules was dropped with a dropper to the antiserum diluents in every line. The microplate was thoroughly shaken. After the antigen was sufficiently mixed with the antisera, the microplate was allowed to stand for 3 hours at room temperature.

Thereafter, the precipitation pattern was read to interprete positive or negative. In this evaluation, diagnosis was made positive when microcapsule particles were spread over the entire bottom of the wells by agglutination and negative when microcapsule particles naturally precipitated to the centre of the bottom of the wells.

For purpose of comparison, commercially available red cells from sheep were treated in a similar fashion and egg albumin was likewise bound to the so treated red cell as an antigen. Positive or negative interpretation was made as described above.

For purpose of further comparison, a polystyrene latex having an average particle size of 0.8 μ and a specific gravity of 1.05 was treated in a similar fashion and egg albumin was likewise bound to the so treated polystyrene latex as an antigen. Diagnosis was made as described above.

The above runs were performed for 10 cases each. The results show that, when using sheep red cells, agglutination sensitivity was equal to that when using microcapsules but non-specific reaction was noted in 2 out of 10 cases; when using the polystyrene latex, non-specific reaction was not noted but agglutination sensitivity was 1/16 of that when using microcapsules. No non-specific reaction was noted when using microcapsules.

As is clearly understood, the system of using microcapsules as carrier particles provided high agglutination sensitivity due to the strong binding capability of the microcapsules to the antigen, and had no chance of causing non-specific reaction as in sheep red cells due to the absence of naturally occurring antigen or antibody therein so that reproducibility was markedly high.

Example 2

In a manner similar to Example 1, RA-factor was measured except that modified human γ-globulin was used as an antigen. The experiment was performed for 10 cases.

The results indicated that when using sheep blood cells, agglutination sensitivity was equal to that when microcapsules but non-specific reaction was noted in 3 out of 10 cases; when using the polystyrene latex, non-specific reaction was not noted, but agglutination sensitivity was reduced to 1/8 that when using microcapsules; whereas non-specific reaction was not noted when using microcapsules.

It is thus clearly understood that the system of using microcapsules as carrier particles provides high agglutination sensitivity due to the strong binding capability of antigen to the microcapsules and has no chance to cause non-specific reaction due to inherent absence of any antibody or antigen, unlike sheep red cells, so that reproducibility is markedly high.

Example 3

A test was performed in a manner similar to Example 1 except that a TP antigen was used as an antigen, and a Treponemal antibody was measured. The experiment was performed for 10 cases.

The results showed that, when using sheep red cells, agglutination sensitivity was equal to that when using microcapsules but non-specific reaction was noted in 3 out of 10 cases; when using the polystyrene latex, non-specific reaction was not noted but agglutination sensitivity was reduced to 1/16 that when using microcapsules; whereas no non-specific reaction was noted in the case of using microcapsules.

As is clearly understood, the system of using microcapsules as carrier particles provides high agglutination sensitivity due to the strong binding capability of microcapsules to the antigen and has no chance to cause any non-specific reaction due to inherent absence of antibody or antigen, unlike sheep red cells, so that reproducibility is also markedly high.

Examples 4 to below show that agglutination sensitivity can be changed by choosing different wall materials for the microcapsules.

Example 4

In 25 g. of an oil mixture (specific gravity of about 1.10) of 11.8 g. of diisopropylnaphthalene and 13.2 g. of chlorinated parrafin (chlorination degree 50%), 0.1 g. of an ethylene diamine/propylene oxide addition product was dissolved. The solution was ice-cooled. In this solution, 4 g. of a 50% methyl ethyl ketone solution of Desmodure-L (tradename, manufactured by Bayer A.G.; adduct of tolylene diisocyanate and trimethylol propane) was dissolved. The resulting oily solution was poured into 65 g. of a 5% aqueous solution of polyvinyl alcohol (saponification degree, 88%; polymerization degree, 500). The mixture was emulsified with stirring. After an average drop size reached about 7 μ, the emulsion was diluted with 100 g. of water. The mixture was reacted at 75°C. for 1 hr. to microencapsulate.

The thus obtained polyurethane-walled microcapsules were washed with a phosphoric acid buffer having the same composition as used in Example 1. Thereafter, 0.5 g. of the thus washed microcapsules were dispersed in 5 ml. of the phosphoric acid buffer. To the dispersion, 1 ml. of egg albumin (25 mg/ml) was added as an antigen. After 100 μl of a 25% aqueous glutaraldehyde solution was further added to the dispersion, the mixture was reacted at room temperature for 1 hr. followed by centrifuge. The precipitate formed was washed with the phosphoric acid buffer. Again, 0.5 g. each of the so washed samples was disposed in 5 ml. of the phosphoric acid buffer to obtain the buffer containing 1% of antigen-bound microcapsules.

Serial dilutions of rabbit antisera up to 2^{12}(4096) times were prepared in a manner similar to Example 1.

Thereafter, 25 μl of the antigen-bound microcapsules (diluted to a 1% concentration) was dropped on the anti-albumin diluents in respective 12 lines on a microplate. The microplate was thoroughly shaken to sufficiently mix the antigen and anti-albumin. The system was allowed to stand at room temperature.

The precipitation patterns were observed and the minimum antibody concentration (antibody detection sensitivity) at which specific agglutination occurred was determined. The results are shown in Table 1 below.

Example 5

To 25 g. of a 10% aqueous solution of carboxy-modified polyvinyl alcohol (molecular weight 100,000, saponification degree 90%, carboxy group-content 5–6%), 2.5 g. of urea, 0.25 g. of resorcine and 0.3 g. of ammonium chloride were added. The mixture was stirred to dissolve. The pH of the resulting solution was adjusted to 4.0 by adding a 0.1N aqueous hydrochloric acid solution thereto. While vigorously stirring, 25 g. of the oil mixture having a specific gravity of 1.10 as used in Example 4 was emulsified in the above-described aqueous solution to prepare an O/W emulsion. The drop size was adjusted to a diameter of about 7 μ. To the resulting emulsion, 6.4 g. of a 37% aqueous formaldehyde solution was added. After the temperature of the system was adjusted to 60°C, encapsulation was performed for further 2 hrs. The polyurea-walled microcapsules thus prepared were washed three times with the phosphoric acid buffer as used in Example 1 to remove the remaining formalin, urea, protective colloid, etc.

Thereafter, the antigen was bound to the microcapsules in a similar fashion to Example 1 and the detection sensitivity of antibody was determined.

Example 6

Polyurethane-urea walled-microcapsules were prepared in a similar fashion to Example 4 except that when the average drop size reached about 7 μ, 100 g. of a 1% aqueous hexamethylene diamine solution was added instead of diluting with 100 g. of water.

The thus prepared polyurethane-urea walled-microcapsules were washed three times with the phosphoric acid buffer as used in Example 1. Then, the antigen was bound to the microcapsules

in a similar fashion to Example 1 and the detection sensitivity of antibody was determined. The results are also shown in Table 1 below.

Table 1

Example No.	Wall Material	Sensitivity for Detecting Antibody
4	polyurethane	2^{11}
5	polyurea	2^{9}
6	polyurethane-urea	2^{9}
1	gelatin	2^{6}

As is clearly understood from the results shown above, the antigen-bound microcapsules obtained with polyurethane walls, polyurea walls and polyurethane-urea walls selectively exhibit an extremely high sensitivity for detecting antibodies. Comparing this with the sensitivity for detecting antibody in the system of using the gelatin wall capsule obtained in Example 1, the sensitivity of the gelatin walled microcapsule indicates 2^{6}, whereas the polyurea and polyurethane-urea walled microcapsules provide a detection sensitivity 8 times that of the gelatin wall microcapsule and the polyurethane walled microcapsule gives the detection sensitivity 32 times that of the gelatin walled microcapsule.

The following examples 7 through 11 and comparison examples 1 through 3 are to demonstrate that microcapsules having a selected range of a specific gravity can improve the time period necessary for judgement of results (hereafter simply referred to as judgement time).

Example 7

To a 10% aqueous solution (25 g.) of a maleic anhydride-methyl vinyl ether copolymer (tradename "GANTREZ-AN 139", molecular weight, ca. 25,000, manufactured by General Aniline & Film Co., Ltd.), 2.5 g. of urea, 0.25 g. of resorcine and 0.3 g. of ammonium chloride were added. The mixture was dissolved under stirring. The resulting solution was adjusted to pH 4.0. An oil mixture of 11.8 g. of diisopropylnaphthalene and 13.2 g. of chlorinated parrafin (chlorine content, 50%) was emulsion-dispersed into the above aqueous solution to form an O/W emulsion. When the average drop size became about 7 μ, stirring was stopped. To the resulting emulsion, 6.7 g. of a 37% aqueous formaldehyde solution and 25 g. of water were added. After the temperature of the system was raised from ambient temperature to 65°C., the reaction was continued for 2 hrs. The thus obtained microcapsules having a specific gravity of about 1.10 were washed three times with a phosphoric acid buffer having the same composition as used in Example 1 to remove the remaining formalin, protective colloid, etc. 0.5 g. of the thus washed microcapsules was taken out and dispersed again in 5 ml. of the phosphoric acid buffer. To the dispersion, 1 ml. of an aqueous solution of egg albumin (25mg/ml) (manufactured by Biochemical Industry Co., Ltd.) was added as an antigen, and 100 μl of a 25% aqueous glutaraldehyde solution was then added to the mixture. Subsequently, the mixture was reacted for 1 hr. at ambient temperature. After centrifuging the system was washed with the phosphoric acid buffer solution 0.5 g. of the microcapsules was again dispersed in 5 ml. of the phosphoric acid buffer.

Examples 8–11 and Comparison Examples 1–3

In a manner similar to Example 7, microcapsules having various specific gravities and antigen-bound microcapsules using such microcapsules were prepared except that the composition of the core oil material (mixing ratio of diisopropylnaphthalene/chlorinated paraffin) was changed as indicated in Table 2 below. As Comparison Example 3, sheep red cells, (specific gravity of about 1.10) was used as a carrier.

Table 2

	Ex. 8	Ex. 9	Ex. 10	Ex. 11	Comp. Ex. 1	Comp. Ex. 2
diisopropyl-naphthalene(g)	14.6	11.1	7.6	6.6	15.6	5.9
chlorinated parrafin(g)	10.4	13.9	17.4	18.4	9.4	19.1
specific gravity	1.07	1.11	1.15	1.16	1.06	1.17

Interpretation of Particulate Agglutination due to Antibody-Antigen Reaction:

Onto serial dilutions of rabbit antibody (positive samples) and antibody-free serial dilutions (negative samples) on a microplate, 25 μl of the thus prepared 1% antigen-bound microcapsules was droped with a dropper, respectively. The microplate was thoroughly shaken to mix. While allowing the plate to stand at room temperature, the judgement time was measured.

Further, observation was made as to if the positive samples and negative samples properly caused agglutination and precipitation respectively, in order to judge (+) or (–). Then, judgement ability was examined by whether the judgement on the positive and negative samples was clear or unclear.

The results obtained are summarized in Table 3 below.

Table 3

	Example No.					Comparison Example No.		
	7	8	9	10	11	1	2	3
specific gravity	1.10	1.07	1.11	1.15	1.16	1.06	1.17	1.10
judgement time(hr.)	ca.2	ca.5	ca.1	<1	<1	ca.10	impossible to inter- prete	ca.4
judgement ability	Δ	Δ	o	o	Δ	x	x	o
positive sample	+	+	+ +	+ +	+	+	–	+ +
negative sample	–	–	––	––	–	+	–	––
total judgement	o	o	◎	◎	o	x	x	o

In judgement ability, o: (+) or (–) can easily be interpreted.
Δ: (+) or (–) can insufficiently but practically interpreted.
x: (+) or (–) cannot be interpreted.
(+) ... Agglutination occurs.
(–) ... Precipitation occurs.

In total judgement, ◎ Detection efficiency is excellent.
o: Detection is possible.
x: Detection is impossible.

As is understood from the results shown above, the antigen-bound microcapsules having a specific gravity ranging from 1.07 to 1.16 are excellent in judgement time although the microcapsules having a specific gravity of 1.07 are somewhat inferior to sheep red cells; that is, judgement time when using the microcapsules having such a specific gravity range is short (approximately 1 to 2 hrs.). In particular, the microcapsules having a specific gravity ranging from 1.11 to 1.15 are preferred in that detection capability thereof is comparable with sheep red cells and further judgement time is very short. In addition, the microcapsule carrier system of the present invention gives no chance of causing undesired non-specific reaction and provides extremely high reproducibility. It is thus concluded, considering these results from an examination accuracy viewpoint, the antigen- or antibody-bound microcapsules having a specific gravity ranging from 1.07 to 1.16 are superior for detecting an antigen or antibody, to sheep red cells. Further, the antigen- or antibody-bound microcapsules having a specific gravity ranging from 1.11 to 1.15 provide greatly improved detection.

The following examples 12 to 14 demonstrate how the microcapsule system of the present invention can be labelled with marking substances thereby enabling quantitative determination of antigen-antibody reactions.

Example 12

To 25 g. of a 10% aqueous solution of a maleic anhydride methyl vinyl ether copolymer (GANTREZ-AN 139, molecular weight, ca. 25,000, manufactured by General Aniline & Film Co., Ltd.), 2.5 g. of urea, 0.25 g. of resorcine and 0.3 g. of ammonium chloride were added. The resulting mixture was stirred to dissolve. To the thus obtained aqueous solution, 26 g. of a mixture of 1.0 g. of White Flour Blue (tradename, manufactured by Sumitomo Chemical Co., Ltd., CI Fluorescent Brightening Agent 91 described in *Senryo Binran* (Handbook of Dye), published Maruzen Co., Ltd., Tokyo, 1974) as a marking substance, 11.8g. of diisopropylna-

phthalene and 13.2 g. of chlorinated parrafin (chlorine content 50%) was added. The mixture was emulsified until the average drop size reached 6 μ. Then, 6.4 g. of a 37% formaldehyde aqueous solution was added to the system. The mixture was heated at 60°C. for 2 hrs. The thus formed microcapsules were washed twice with water 1.0 g. of the capsules was taken and suspended in 10 ml of a phosphoric acid buffer having the same composition as used in Example 1. Subsequently, 100 μl of a 25% aqueous glutaraldehyde solution was mixed with the suspension. The mixture was then reacted at 25°C. for 1 hr. After centrifuging, the system was washed with the phosphoric acid buffer.

Separately, 0.95 ml. of physiological saline solution was added to 0.05 ml. of anti-human IgG (28 mg Ag/ml) to prepare a solution mixture. The solution mixture was mixed with 2 ml. (0.25% concentration) of the glutaraldehyde-treated microcapsules described above. The mixture was then incubated at 37°C. for 1 hr. and thereafter allowed to stand in a refrigerator overnight. After the system was washed twice with a 0.2% glycine-containing physiological saline solution, the phosphoric acid buffer further containing 1 ml. of 3% BSA was added to the system. Thus, a reagent comprising microcapsules having bound thereto anti-human IgG was obtained.

In order to determine a Treponemal antibody blood, a TP antigen (Treponema pallidum (Nicholas strain)) was pulverized with an electric wave of 25 KHz. This antigen was coated onto a glass plate and dried and then immobilized with acetone. On the coated area, 25 μl of diluted serum was dropped. The reaction was performed at 37°C. for 1 hr. After washing with the phosphoric acid buffer, the above-described reagent was added to the reaction mixture, the reaction was performed at 37°C. or further 1 hr. A fluorescent intensity was measured using a fluorometer. Thus, the pallidum antigen could easily be determined accurately.

Example 13

Microcapsules were obtained in a manner similar to Example 12, except that Fe_2O_3 was incorporated in the core material. After the microcapsules were treated with glutaraldehyde in a manner similar to Example 12, an anti-α-fetoprotein was bound thereto.

The so obtained microcapsules were employed as a solid phase carrier in an enzyme immunology sandwich method and the amount of α-fetoprotein in a serum was determined. In this system, the carrier could easily be separated from the solution phase and measurement procedure was markedly simplified and reproducibility as well as stability were also improved, as compared to the prior art system where glass beads were employed as a carrier.

Example 14

Microcapsules were prepared in a similar to Example 12 except that 2–hydroxy–4–methoxybenzophenone was incorporated in the core material. After treatment with glutaraldehyde in a manner similar to Example 12, an anti-HCG was bound thereto and the amount of HCG in a sample could be detected by UV absorption of 280 to 340 nm in a simple manner.

Example 15

Microcapsules having a specific gravity of 0.80 were prepared in a manner similar to Example 7 except that 18.0 g. of isoparaffin containing 1% oleophilic blue dye 0–79 (CI Solvent Blue 55, *Senryo Binran* (Handbook of Dye), Maruzen Co., Ltd., 1974) was included in the core material.

Egg albumin was bound to the thus obtained microcapsules in a manner similar to Example 7.

25 μl of the thus prepared 1% antigen-bound microcapsules was dropped onto whole blood serial dilutions containing rabbit antibody (positive samples) and antibody-free whole blood serial dilutions (negative samples) on a microplate with a dropper. The microplate was well shaken to mix. After allowing them to stand for 10 mins. at ambient temperature, the blood cells were precipitated and the microcapsules were floated on the surface of the supernatant serum phase, where, presence or absence of agglutination was interpreted by the eye or microscopically. The interpretation was very easy because of the colored microcapsules and floatation property of the microcapsules due to a small specific gravity. (This enables the use of whole blood).

For preventing blood coagulation, heparin was incorporated into the whole blood as an anti-coagulant.

CLAIMS

1. A microcapsule comprising a core material and a wall material having an antigen or antibody bonded to the surface thereof.

2. A microcapsule according to claim 1 having a specific gravity between about 0.8 and about 1.20.

3. A microcapsule according to claim 2 having a specific gravity between about 1.05 and about 1.20.

4. A microcapsule according to claim 3 having a specific gravity between about 1.07 and

about 1.16.

5. A microcapsule according to any one of claims 1 to 4 wherein the wall material is a polyurethane, a polyurea or a polyurethane-urea.

6. A microcapsule according to any one of claims 1 to 5 wherein one or more of said core material, wall material, antigen and antibody is labelled with a marking substance.

7. A microcapsule according to claim 6 wherein the marking substance is an isotope, an enzyme, a fluorescent substance, a magnetic substance, a UV absorent or a dye.

8. A microcapsule according to claim 7 wherein the marking substance is an isotope and is I^{125} or I^{131}.

9. A microcapsule according to claim 7 wherein the marking substance is an enzyme and is an alkali phosphate, β-galactosidase, acetylcholine esterase, glucoamylase, maleic acid dehydrogenase, glucose–6–phosphoric acid dehydrogenase, peroxidase and glutaroxidase.

10. A microcapsule according to claim 7 wherein the marking substance is a fluorescent substance and is stilbene or a derivative thereof, a coumarine derivative, a pyrazoline derivative, a benzoxazole derivative, bisoxazole, or 4,4'–bistriazinyl.

11. A microcapsule according to claim 7 wherein the marking substance is a magnetic substance and is iron, nickel, cobalt, CrO_2, CoO, NiO, Mn_2O_3, a magnetic zinc oxide, a magnetic iron oxide, an alloy of Al, Ni, Co or Cu, chalcogenide or ferricolloid powder.

12. A microcapsule according to claim 7 wherein the marking substance is a UV absorbant and is a salicylic acid derivative, a benzophenone derivative or a benzotriazole derivative.

13. A microcapsule according to any one of claims 7 to 12 wherein the marking substance is located outside the wall material.

14. A microcapsule according to any one of claims 7 to 12 wherein the marking substance is incorporated in the core material.

15. A microcapsule as claimed in claim 1, substantially as described in any of the Examples herein.

16. A method making a microcapsule as claimed in claim 1 conducted substantially as described in any of the Examples herein.

17. A method for determining a biological component having immunological activity which comprises mixing a microcapsule comprising a core material and a wall material and having an antigen or antibody bonded on the surface thereof with said biological component thereby and causing immunological reaction therebetween.

18. A method as claimed in claim 17 wherein said microcapsule have a specific gravity between 0.8 and 1.20.

19. A method as claimed in claim 17 or claim 18 wherein the microcapsule wall is composed of a polyurethane, a polyurea or a polyurethane-urea.

20. A method as claimed in any one of claims 17 to 19 wherein the microcapsule, the antigen and/or the antibody is labelled with a marking substance.

21. A method as claimed in claim 17, conducted substantially as described in any of the Examples herein.

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